Immunocytochemistry of Tubulin in Spermatozoa of Platyhelminthes

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ABSTRACT

Indirect immunofluorescence and electron microscope post-embedding immunocytochemistry were used to detect tubulin in spermatids and spermatozoa of various Platyhelminthes. General sperm morphology is exemplified by conventional electron microscope observations on *Gorgodera* sp. and *Haematoloechus* sp. The utility of tubulin indirect immunofluorescence for the understanding of the filiform spermatozoa of Platyhelminthes is underlined by a comparison between *Echinostoma* (Digenea) and *Symsagittifera* (Acoela). This technique might be useful for further comparative research with phylogenetic implications. In the digenean *Echinostoma* sp., alpha- and beta-tubulin are detected in the doublets of the 9+"1" axonemes and in the cortical singlet microtubules, but alpha-acetylated-tubulin is present in the axonemal doublets and absent in the cortical microtubules. The central core of the Platyhelminthes 9+"1" axoneme is not labelled by any of the three monoclonal anti-tubulin antibodies used (anti-alpha-, anti-beta- and anti-alpha-acetylated-tubulin).

RÉSUMÉ

Immunocytochimie de la tubuline dans les spermatozoïdes de Plathelminthes

L'immunofluorescence indirecte et l'immunocytochimie ultrastructurale post-inclusion ont été utilisées pour détecter la tubuline dans les spermatides et les spermatozoïdes de Plathelminthes variés. La morphologie générale des spermatozoïdes est explicitée par des observations de microscopie électronique conventionnelle de *Gorgodera* sp. et *Haematoloechus* sp. L'intérêt de l'immunofluorescence indirecte de la tubuline pour la compréhension de la structure des spermatozoïdes filiformes des Plathelminthes est souligné par une comparaison entre *Echinostoma* (Digenea) et *Symsagittifera* (Acoela). Cette technique pourrait être utile pour une recherche comparée à but phylogénique. Chez *Echinostoma* sp., l'alpha-tubuline et la beta-tubuline sont détectées dans les doublets des axonèmes 9+"1" et dans les singulets corticaux, mais la tubuline acétylée est présente seulement dans les doublets des axonèmes et absente dans les singulets corticaux. L'élément central des axonèmes 9+"1" de Plathelminthes n'est marqué par aucun des trois anticorps utilisés (contre la tubuline alpha, beta, et alpha-acétylée).

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Source : MNHN, Paris

Tubulin, the protein constituting the microtubules, is one of the major proteins in spermatozoa which have axonemes and/or microtubules. Tubulin shows a high degree of heterogeneity owing to two factors, genetic diversity [21] and posttranslational modifications: acetylation [19], glutamylation [8], detyrosylation [33], phosphorylation [10, 22] and the recently discovered polyglycylation [28]. Because tubulins are well conserved during evolution [1] and posttranslational modifications widely distributed among various species, antibodies raised against tubulin or posttranslational modifications of one species generally recognize the same epitope in other species and can thus be used as tool to investigate tubulin properties in many systems.

In this study, we demonstrate that different anti-tubulin antibodies can differentiate distinct subcellular population of microtubules in spermatozoa of Platyhelminthes. In addition, immunocytochemistry of tubulin gives valuable information about the general morphology of the spermatozoon, and new insight into the composition of the 9+"1" axoneme of the Platyhelminthes. These results might be of phylogenetic interest when further comparative data are obtained.

MATERIAL AND METHODS

Material. Digeneans were collected from hosts which can be kept easily in the laboratory. *Gorgodera* sp. and *Haematoloechus* sp. were collected from commercial frogs (*Rana* sp.) originating from various localities of Eastern Europe. *Echinostoma liei* and *Echinostoma caproni* were collected from experimentally infested laboratory mice. The Acoela *Symsagittifera schultzei* was extracted from sand collected in Roscoff, France.

Light microscope immunocytochemistry. Germ cells were obtained by squashing worms in a drop of PBS (phosphate buffer saline, Sigma) on a pit slide previously washed with alcohol and acetone. Slides were allowed to dry for 1 h under a fan, then kept at 4°C and processed within 24 h. Cells were permeabilized in acetone (10 min) and rinsed (PBS, 3 x 5 min). Non-specific antigens were blocked with 2% Bovine Serum Albumin (Sigma) in PBS (BSA-PBS) for 45-90 min at room temperature. A monoclonal anti-tubulin antibody (anti-alpha-tubulin, clone DM 1A, Sigma, or anti-beta-tubulin, clone TUB 2.1, Sigma or anti-acetylated-tubulin, clone 6-11B-1, Sigma [26]) diluted at 1/200 to 1/600 in BSA-PBS was applied for 40 min at room temperature. After rinsing (PBS 3 x 5 min), a FITC-conjugated antibody (Goat anti-mouse, Nordic, 1/40 in PBS) was applied for 40 min at room temperature. For double labelling, supplementary steps were added as follows: anti-tubulin polyclonal antibody (Sigma, ref. T-3526) diluted at 1/40 in BSA-PBS applied for 40 min at room temperature, rinsed (PBS 3 x 5 min) and followed by a TRITC-conjugated antibody (Goat anti-rabbit, Nordic, 1/40 in PBS) for 40 min at room temperature. The nuclear dye Hoechst 33258 (1 µg/ml in PBS, 10 min) or DAPI (1 µg/ml, 10 min) was sometimes used for labelling the nucleus. After a final rinse (PBS 3 x 5 min), mounting was done in Citifluor (Citifluor Ltd, London, UK) and slides were sealed with nail enamel. Controls were done by omitting the first antibody or by using a non-relevant mouse antibody. Observations were made with a Nikon Optiphot epifluorescence microscope equipped with a mercury lamp and a single band Nikon filter for FITC channel (B-2A), TRITC channel (G-2A) or Hoechst channel (UV-1A), or a double-band (FITC/TRITC) Omega filter (XF 52).

Conventional electron microscopy. Living specimens cut into pieces were fixed for 1 h in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate at pH 7.2 at 4°C. After rinsing in the same buffer, the worms were postfixed for 1 h in 1% osmium tetroxide in the same buffer, dehydrated in ethanol and propylene oxide, and embedded in SPURR's medium or in Epon. Ultrathin sections were contrasted with DADDOW's method [7] or with conventional lead citrate and uranyl acetate, and observed with a Hitachi H600 electron microscope.

Post-embedding electron microscope immunocytochemistry. Living worms were fixed for 1 h in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate at pH 7.2 at 4°C. After rinsing in the same buffer (3 x 10 min), the worms were embedded in LR White resin (medium grade). The resin was polymerized in tightly capped gelatin capsules for 10 h at 60°C. Ultrathin sections were placed on nickel or gold grids. Grids were rinsed (PBS, 3 x 5 min), and non-specific antigens were blocked with goat immunoglobulins (Sigma) diluted at 1/30 in PBS for 1h. A monoclonal anti-tubulin antibody (anti-alpha-tubulin, clone DM 1A, Sigma, or anti-beta-tubulin, clone TUB 2.1, Sigma or anti-acetylated-tubulin, clone 6-11B-1, Sigma [26]) diluted at 1/100 in PBS, was applied for 40 min at room temperature. After rinsing (PBS, 3 x 5 min), a gold-conjugated antibody (Goat anti-mouse, 15 nm gold beads, 1/20 in PBS) was applied for 1 h at room temperature. After a final rinse (PBS 3 x 5 min, then distilled water, 3 x 5 min), the grids were dried on Whatman paper, stained with DADDOW's method [7] and observed with a Hitachi H600 electron microscope.

Source : MNHN, Paris

RESULTS

Conventional electron microscopy (Fig. 1)

Results of conventional electron microscopy are briefly presented in this paper to show the structure described below with electron microscope immunocytochemistry. In the digeneans, spermiogenesis is homogeneous in most species [16, 17]. The spermatid shows a protrusion called the zone of differentiation (Fig. 1a). At the distal extremity of this zone of differentiation, three processes elongate: a median cytoplasmic process, and two flagella. The median cytoplasmic process contains singlet microtubules aligned below the membrane (Fig. 1b). Each flagellum contains an axoneme with the 9+"1" trepaxonematan structure (Fig. 1c). The spermatozoon (Fig. 1c) is later produced by the fusion of the three processes, and contains two 9+"1" axonemes, dorsal and ventral microtubules, an elongate mitochondrion and an elongate nucleus. The 9+"1" axonemes (Fig. 1b, c) do not contain the pair of central microtubules of the almost ubiquitous 9+2 axonemes but have, instead, a solid central core.

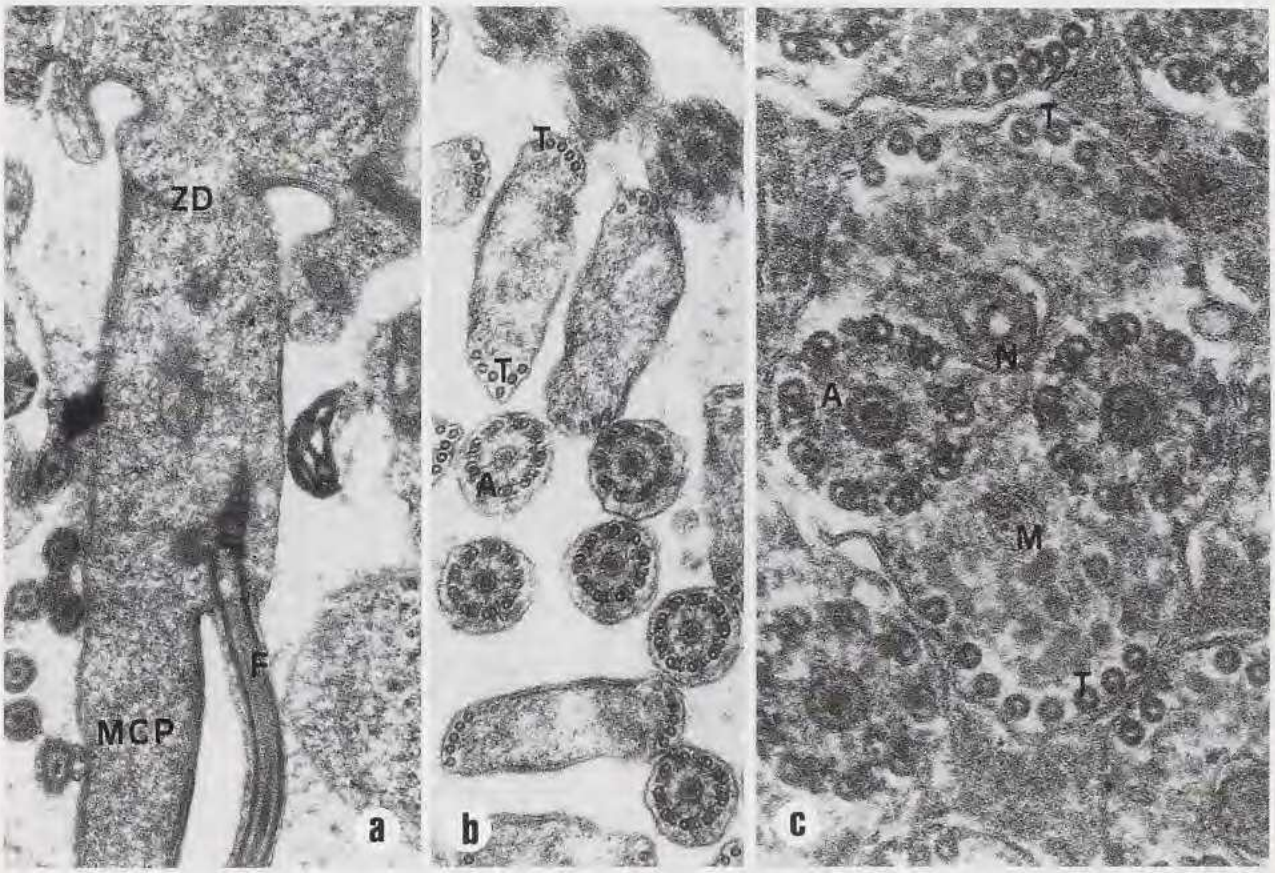

FIG. 1. — Conventional electron microscopy of digenean spermatozoa. **a, b:** *Gorgodera* sp. **a:** Zone of differentiation (ZD) in spermatid, longitudinal section showing median cytoplasmic process (MCP) and flagella (one sectioned here, F) which will fuse to produce the mature spermatozoon. **b:** Median cytoplasmic process with singlet microtubules (T) and free flagella containing 9+"1" axonemes (A), transverse section. **c:** *Haematoloechus* sp., mature spermatozoon, transverse section. A, axonemes with 9+"1" trepaxonematan pattern; M, mitochondrion; N, nucleus; T, dorsal and ventral microtubules. a, x 20 000; b, x 48 000; c, 125 000.

Source : MNHN, Paris

Light microscope immunocytochemistry (Fig. 2)

Digenean spermatozoa, in all species studied, are long, filiform cells. Spermatozoa contain two parallel axonemes. These are labelled by the anti-tubulin antibodies (Fig. 2a) and appear as two closely parallel lines in some cases, but often as a single thick line. In some cases, the process of drying and re-hydrating during processing partly alters the cell and, in certain regions of the spermatozoon, two lines, sometimes widely separate, are visible. The use of various antibodies and the technique of double labelling (Fig. 2b, c) reveal that tubulin epitopes are different in these two microtubular structures. The two lines are labelled by the polyclonal anti-tubulin (Fig. 2b), the anti-alpha-tubulin (Fig. 2a) and the anti-beta-tubulin monoclonal antibodies, but the monoclonal anti-acetylated-alpha-tubulin antibody labels only one line (Fig. 2c). This shows that two distinct subpopulations of microtubules are present, but interpretation of the nature of these two lines requires the use of electron microscope immunocytochemistry, described below.

Acoel spermatozoa also contain two parallel axonemes, which do not show the 9+"1" pattern but instead a 9+0 or 9+2 pattern [27]. Immunocytochemistry (Fig. 2d) reveals that these axonemes are widely separated and show longitudinal undulations. The axonemes are visible with the three antibodies used.

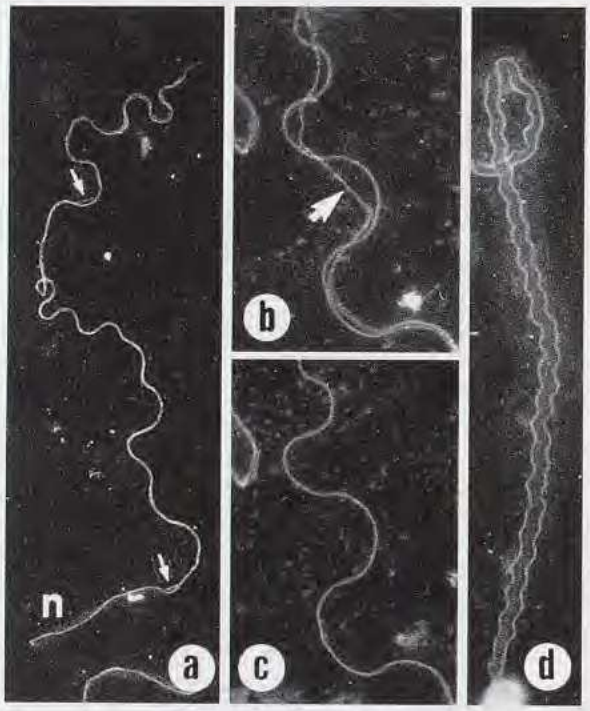

FIG. 2. — Light microscope immunocytochemistry of platyhelminth spermatozoa. **a-c:** *Echinostoma caproni* (Digenea). **a:** View of spermatozoon showing filiform pattern. Anti-alpha-tubulin labelling. n, nucleus (evidenced by a nuclear dye, not shown here). Two lines are visible in limited regions of the cell (arrows). **b, c:** double labelling with polyclonal anti-tubulin antibody (b) and monoclonal anti-acetylated-alpha-tubulin antibody (c) in a region where two lines are visible. Acetylated-alpha-tubulin is detected in one line only. Interpretation of these labelling requires electron microscope immunocytochemistry (see Fig. 3 and Discussion). **d:** *Symsagittifera schultzei* (Acoela), anti-alpha-tubulin antibody. The two axonemes are widely separated and undulate. a, d, x 600; b, c, x 1 200.

Electron microscope immunocytochemistry. (Fig. 3)

Axonemal microtubules and cortical singlet microtubules show striking differences in their labelling. The anti-alpha tubulin and the anti-beta-tubulin antibodies label the cortical singlets together with the axonemal microtubules (Fig. 3a, b, d, e). The anti-acetylated-alpha-tubulin antibody (Fig. 3c, f, g) labels only the axonemal microtubules ; the cortical singlets are not labelled.

None of the antibodies labels the central core of the 9+"1" axoneme (Fig. 3a-g).

Source : MNHN, Paris

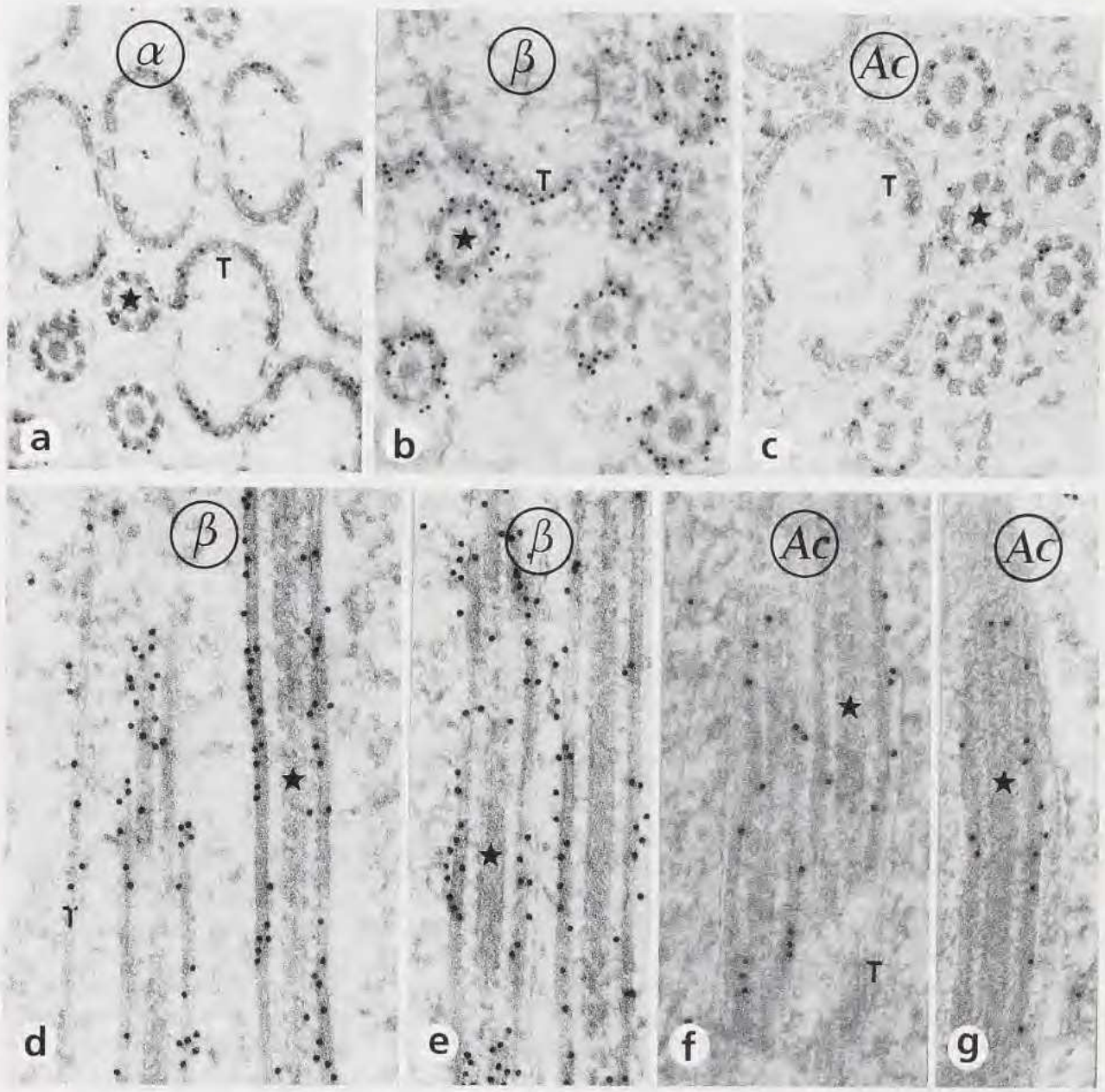

FIG. 3. — Electron microscope immunocytochemistry of spermatids of *Echinostoma liei* (Digenea). **a-c:** Median cytoplasmic process and flagella, transverse section (compare with figure 1b). **a:** anti-alpha-tubulin. **b:** anti-beta-tubulin. **c:** anti-acetylated-alpha-tubulin. **d-g:** Spermatids, longitudinal section. **d, e:** anti-beta-tubulin; **f, g:** anti-acetylated-alpha-tubulin. Axonemal microtubules are labelled in each case, but singlet microtubules (T) in the median cytoplasmic process are labelled by the anti-alpha and anti-beta-tubulin antibodies but not by the anti-acetylated-alpha-tubulin antibody. The central core (star) of the 9+"1" axoneme is never labelled by the anti-tubulin antibodies. a-c, x 50 000; d-f, 70 000; g, x 60 000.

Source : MNHN, Paris

DISCUSSION

Tubulin immunocytochemistry: an innovative technique for understanding the general morphology of spermatozoa

Spermatozoa of Platyhelminthes are generally very long and filiform [16, 17], without a "head" and a "tail", and thus are chiefly described by means of cross sections. Ordering the various cross sections along a scheme of the sperm body from random sections is not trivial and serial sectioning is very time consuming. Anti-tubulin immunocytochemistry, although a routine technique in cell biology, has not previously been used for purely morphological studies. This technique allows a view of the whole filiform spermatozoon and of the arrangement of the microtubular organelles along its length. The two examples presented in Figure 2 show the differences between a "turbellarian" spermatozoon, here an acoelan, with almost independent axonemes which undulate along the cell, and a digenean, with very close axonemes. Anti-tubulin immunocytochemistry is an innovative technique for the understanding of spermatozoa in two very different cases: filiform spermatozoa which are almost "two-dimensional" such as those of Platyhelminthes (this study), and complex three-dimensional spermatozoa such as those of crustaceans [24, 25, 34].

Tubulin subpopulations in Platyhelminthes spermatozoa: axonemes versus cortical microtubules

The results of electron microscope immunocytochemistry allows the interpretation of the double labelling observed in *Echinostoma* with indirect immunofluorescence (Fig. 2b, c) as follows: one line, labelled by all the antibodies used, is made up of the two closely associated axonemes; the other line, labelled by all antibodies except the anti-acetylated-alpha-antibody, represents the singlet microtubules.

The location of tubulin in Platyhelminthes has received little attention [32]. Results presented here show that different tubulin epitopes are located in the axonemes and in the cortical microtubules. The detection of alpha and beta-tubulin in the axonemes was expected, since alpha and beta tubulin are constituents of the microtubules. It, however, reveals that anti-tubulin antibodies developed against non-Platyhelminthes antigens can be used for this purpose.

The differential location of acetylated tubulin (present in axonemes, absent in cortical microtubules) is a more interesting finding. Posttranslational varieties of tubulin have been investigated in spermatozoa of only a few species: glutamylated in mammals [11, 18], tyrosinated in sea urchin, man [13] and rat [15], polyglycylated in *Drosophila* [2]. Acetylated tubulin has been detected in male germ cells of sea urchin [26], insects [26, 35], fish [20], and various mammals including man [11, 12, 15], but is apparently absent in the transient microtubules of aflagellate nematode spermatids (MANSIR & JUSTINE, this volume) [23]. In mammals, a subcellular partition of acetylated tubulin has been found: it is present in axonemes, but not in the singlet microtubules of the manchette [11, 12, 15]. The finding reported here show a similarity between mammalian spermatids and platyhelminth spermatozoa, both having acetylated axonemes and non-acetylated cortical microtubules.

The absence of tubulin in the central core of the 9+"1" axoneme

The 9+"1" axonemal structure is a synapomorphy for the Trepaxonemata [9]. Although the 9+"1" axoneme has been the subject of detailed studies with various methods of electron microscopy [3-6, 14, 29-31], the chemical nature of the central core has not been investigated. This study is, as far as we know, the first attempt to characterize the proteins present in the central core. The central core of the 9+"1" axoneme is not labelled by any of the three monoclonal anti-tubulin antibodies used (anti-alpha-, anti-beta- and anti-alpha-acetylated-tubulin), therefore suggesting the absence of tubulin in the central core. The absence of tubulin in the central core, if confirmed, would underline the uniqueness of the 9+"1" trepaxonematan structure in the Animal Kingdom.

Source : MNHN, Paris

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REFERENCES

1. ADOUTTE, A., CLAISSE, M., MAUNOURY, R. & BEISSON, J., 1985. — Tubulin evolution: ciliate-specific epitopes are conserved in the ciliary tubulin of Metazoa. *Journal of Molecular Evolution*, **22**: 220-229.
2. BRESSAC, C., BRÉ, M.-H., DARMANADEN-DELORME, J., LAURENT, M., LEVILLIERS, N. & FLEURY, A., 1995. — A massive new posttranslational modification occurs on axonemal tubulin at the final step of spermatogenesis in *Drosophila*. *European Journal of Cell Biology*, **67**: 346-355.
3. BURTON, P. R., 1967. — Fine structure of the unique central region of the axial unit of lung-fluke spermatozoa. *Journal of Ultrastructure Research*, **19**: 166-172.
4. BURTON, P. R., 1968. — Effects of various treatments on microtubules and axial units of lung-fluke spermatozoa. *Zeitchrift für Zellforschung*, **87**: 226-248.
5. BURTON, P. R. & SILVEIRA, M., 1971. — Electron microscopic and optical diffraction studies of negatively stained axial units of certain platyhelminth sperm. *Journal of Ultrastructure Research*, **36**: 757-767.
6. COSTELLO, D. P., 1973. — A new theory on the mechanics of ciliary and flagellar motility. II. Theoretical considerations. *Biological Bulletin (Woods Hole)*, **145**: 292-309.
7. DADDOW, L. Y. M., 1986. — An abbreviated method of the double lead stain technique. *Journal of Submicroscopic Cytology*, **18**: 221-224.
8. EDDÉ, B., ROSSIER, J., LE CAER, J.-P., DESBRUYÈRES, E., GROS, F. & DENOULET, P., 1990. — Posttranslational glutamylation of alpha-tubulin. *Science*, **247**: 83-85.
9. EHLERS, U. , 1985. — *Das Phylogenetische System der Plathelminthes*. Stuttgart, G. Fischer: .
10. EIPPER, B. A., 1974. — Properties of rat brain tubulin. *Journal of Biological Chemistry*, **249**: 1407-1416.
11. FOUQUET, J.-P., EDDE, B., KANN, M.-L., WOLFF, A., DESBRUYERES, E. & DENOULET, P., 1994. — Differential distribution of glutamylated tubulin during spermatogenesis in mammalian testis. *Cell Motility and the Cytoskeleton*, **27**: 49-58.
12. FOUQUET, J.-P. & KANN, M.-L., 1994. — The cytoskeleton of mammalian spermatozoa. *Biology of the Cell*, **81**: 89-93.
13. GUNDERSEN, G. G. & BULINSKI, J. C., 1986. — Microtubule arrays in differentiated cells contain elevated levels of a post-translationally modified form of tubulin. *European Journal of Cell Biology*, **42**: 288-294.
14. HENLEY, C., COSTELLO, D. P., THOMAS, M. B. & NEWTON, W. D., 1969. — The "9+1" pattern of microtubules in spermatozoa of *Mesostoma* (Plathelminthes, Turbellaria). *Proceedings of the National Academy of Sciences, USA*, **64**: 849-856.
15. HERMO, L., OKO, R. & HECHT, N. B., 1991. — Differential post-translational modifications of microtubules in cells of the seminiferous epithelium of the rat: a light and electron microscope immunocytochemical study. *Anatomical Record*, **229**: 31-50.
16. JUSTINE, J.-L., 1991. — Phylogeny of parasitic Platyhelminthes: a critical study of synapomorphies proposed on the basis of the ultrastructure of spermiogenesis and spermatozoa. *Canadian Journal of Zoology*, **69**: 1421-1440.
17. JUSTINE, J.-L., 1995. — Spermatozoal ultrastructure and phylogeny of the parasitic Platyhelminthes. *In:* B. G. M. JAMIESON, J. AUSIO & J.-L. JUSTINE, *Advances in Spermatozoal Phylogeny and Taxonomy*. *Mémoires du Muséum national d'Histoire Naturelle*, **166**: 55-86.
18. KANN, M.-L., PRIGENT, Y. & FOUQUET, J.-P., 1995. — Differential distribution of glutamylated tubulin in the flagellum of mouse spermatozoa. *Tissue and Cell*, **27**: 323-329.

Source : MNHN, Paris

19. LEDIZET, M. & PIPERNO, G., 1987. — Identification of an acetylation site of *Chlamydomonas* α-tubulin. *Proceedings of the National Academy of Sciences, USA*, **84**: 5720-5724.

20. LESSMAN, C. A., ZHANG, J. & MACRAE, T. H., 1993. — Posttranslational modifications and assembly characteristics of goldfish tubulin. *Biology of the Cell*, **79**: 63-70.

21. LUDUEÑA, R. F., 1993. — Are tubulin isotypes functionally significant. *Molecular Biology of the Cell*, **4**: 445-457.

22. LUDUEÑA, R. F., ZIMMERMANN, H. P. & LITTLE, M., 1988. — Identification of the phosphorylated β-tubulin isotype in differentiated neuroblastoma cells. *FEBS Letters*, **230**: 142-146.

23. MANSIR, A. & JUSTINE, J.-L., 1995. — Centrioles with ten singlets in spermatozoa of the parasitic nematode *Heligmosomoides polygyrus*. *In:* B. G. M. JAMIESON, J. AUSIO & J.-L. JUSTINE, *Advances in spermatozoal phylogeny and taxonomy*. *Mémoires du Muséum national d'Histoire Naturelle*, **166**: 119-128.

24. PEREZ, C., ROCO, M., CASTRO, A., DUPRE, E., SCHATTEN, G. & BARROS, C., 1991. — Localization of microfilaments and a tubulin-like protein in crustacean (*Rhynchocinetes typus*) spermatozoon. *Molecular Reproduction and Development*, **28**: 373–379.

25. PEREZ, R. A., LANGFORD, G. M., ECKBERG, W. R. & ANDERSON, W. A., 1986. — Contractile proteins (actin, myosin) and tubulin are revealed within DNA-containing nucleocytoplasm in mature spermatozoa in *Libinia emarginata*. *Journal of Submicroscopic Cytology*, **18**: 471–480.

26. PIPERNO, G. & FULLER, M. T., 1985. — Monoclonal antibodies specific for an acetylated form of alpha-tubulin recognize the antigen in cilia and flagella from a variety of organisms. *Journal of Cell Biology*, **101**: 2085-2094.

27. RAIKOVA, O. I. & JUSTINE, J.-L., 1994. — Ultrastructure of spermiogenesis and spermatozoa in three Acoels (Platyhelminthes). *Annales des Sciences Naturelles (Zoologie)*, **15**: 63-75.

28. REDEKER, V., LEVILLIERS, N., SCHMITTER, J.-M., LE CAER, J.-P., ROSSIER, J., ADOUTTE, A. & BRÉ, M.-H., 1994. — Polyglycylation of tubulin: a posttranslational modification of axonemal microtubules. *Science*, **266**: 1688-1691.

29. SILVEIRA, M., 1969. — Ultrastructural studies on a "nine plus one" flagellum. *Journal of Ultrastructure Research*, **26**: 274-288.

30. SILVEIRA, M., 1975. — The fine structure of 9+1 flagella in turbellarian Flatworms. *In:* B. A. AFZELIUS, *The Functional Anatomy of the Spermatozoon*. Oxford, Pergamon Press: 289-298.

31. SILVEIRA, M. & PORTER, K. R., 1964. — The spermatozoids of flatworms and their microtubular systems. *Protoplasma*, **59**: 240-264.

32. STITT, A. W., FAIRWEATHER, I., TRUDGETT, A. G. & JOHNSTON, C. F., 1992. — *Fasciola hepatica*: localization and partial characterization of tubulin. *Parasitology Research*, **78**: 103-107.

33. THOMPSON, W. C., 1982. — The cyclic tyrosination/detyrosination of alpha tubulin. *Methods in Cell Biology*, **24**: 235-255.

34. TUDGE, C. C. & JUSTINE, J.-L., 1994. — The cytoskeletal proteins actin and tubulin in the spermatozoa of four decapod crabs (Crustacea, Decapoda). *Acta Zoologica (Stockholm)*, **75**: 277-285.

35. WILSON, P. J. & FORER, A., 1989. — Acetylated alpha-tubulin in spermatogenic cells of the crane fly *Nephrotoma suturalis*: kinetochore microtubules are selectively acetylated. *Cell Motility and the Cytoskeleton*, **14**: 237-250.

Source : MNHN, Paris